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The *Agrobacterium tumefaciens* T pilus composed of cyclic T pilin is highly resilient to extreme environments

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Abstract

Agrobacterium tumefaciens T pili are long semi-rigid, flexuous filaments of 10 nm diameter that are primarily composed of T pilin cyclized protein subunits. The cyclic character of T pilin apparently confers a high level of structural stability on the T pilus. Purified T pili subjected to extreme environmental conditions such as acid and alkali, including glycerol remained relatively unaffected morphologically. T pili lost their semi-rigidity when subjected to high temperatures and high pH, and dissociated into donut shaped subunits when exposed to Triton X-100. Sodium dodecyl sulfate increased the uptake of uranyl acetate exposing a 2 nm wide lumen running the length of the T pilus filament.

Keywords: T pilus; T pilin; VirB2 gene; Cyclic peptide; *Agrobacterium tumefaciens*

1. Introduction

T pili are generated when *Agrobacterium tumefaciens* cells are induced naturally by plant phenolic compounds or experimentally by the addition of an inducer such as acetosyringone. Induction leads to the expression of virulence (*vir*) genes located on the resident Ti plasmid. Of these *vir* genes, the 11 genes of the *virB* operon are involved in the synthesis and assembly of the T pilus, with the product of the *virB2* gene being the subunit precursor of the T pilus [10]. The precursor is the full length VirB2 protein (propilin) that is cleaved into a 7.2-kDa protein both in *Escherichia coli* and in *A. tumefaciens* [8]. The resulting protein of 74 amino acid residues becomes linked by a peptide bond between the amino- and carboxyl-terminal residues to generate a cyclic peptide (the T pilin) [5]. T pilin subunits are transported across the bacterial membranes and assembled into an exocellular, semi-rigid T pilus filament of 10 nm diameter protruding from the bacterial cell [10–12]. The T pilus is thought to provide either a conduit through which the T-DNA–protein complex is transported or a structure for intimate contact between the bacteria and the plant host cell. Whichever may be the functional role of T pili, resiliency of this appendage may play a critical role for efficient virulence [13]. Hence, in the present study, we have tested the durability of the T pilus by observing its structural stability when subjected to extreme environmental conditions.

2. Materials and methods

2.1. Bacterial strain and growth conditions

The wild-type, virulent *A. tumefaciens* strain C58 was used in this study [14]. Strain C58 was grown in medium 523 [10] at 28°C. Induction of strain C58 was carried out at 19°C in I medium containing 200 μM acetosyringone [10].

2.2. Isolation and purification of T pili

T pili were isolated and purified as described previously [10] and included an additional fractionation step using CsCl density gradient centrifugation [5]. Immunoblots

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were prepared according to Lai and Kado [10] using anti-VirB2 antiserum.

2.3. Electron microscopy

T pili preparations negatively stained with 2% uranyl acetate were examined by transmission electron microscopy using a Phillips EM410 electron microscope at 80 kV as described previously [10].

3. Results and discussion

3.1. Factors affecting the structural stability of T pili

When induced, *A. tumefaciens* cells accumulate cyclic T pilin subunits that make their way to the cell surface to form the T pilus [11]. Upon inspection by electron microscopy, the T pilus appears to be a durable filamentous appendage. In support of this premise, we subjected purified T pili to various substances and to extreme environments. As shown in Table 1, T pili treated with either alkali, urea or glycerol resulted in no obvious morphological change. Glycerol is known to depolymerize type 1 and P pili [1,18], yet, glycerol was unable to depolymerize T pili. When T pili were treated with 0.1 N NaOH (pH 13.0), the tip of some filaments partially depolymerized with the formation of a string of bead-like structures (Fig. 1B). A knob-like structure attached to the ends of the T pili is commonly observed (Fig. 1A) and appears like the sacculus-like structure observed by other workers [17]. T pili incubated at 37°C appear unaltered in morphology, while incubation at 70°C causes curling from the pilus tip and down the T pilus filament (Fig. 1C). The curling is exacerbated when T pili are incubated at 70°C at pH 13, giving the impression that the semi-rigid filament becomes relaxed (or melted) under these conditions (Fig. 1D).

Treatment of purified T pili with 0.1% SDS causes electron dense staining along the entire length of the pilus filament (Fig. 1E), revealing a channel of approximately 2.0 nm width (Fig. 1E inset). Full depolymerization of T pili filaments was achieved with 1% Triton X-100. The treatment produces donut shaped structures (Fig. 1F), of which a magnified view reveals an apparently hollow opening in the center of the structure (Fig. 1F inset).

The overall effects of extreme environmental conditions on T pilus structural stability are summarized in Table 1. T pilus subunits are unaltered in molecular mass by most of the treatments, viz., detergents, urea, pH extremes, or glycerol, whereas treatment with proteinase K completely digests the T pilus (Fig. 2, lane 2). These data suggest that the T pilus may be relatively resilient in the natural environment such as in the rhizosphere. The resiliency could be due in part to the compact cyclic peptide composition of the T pilus.

In the assembly of the T pilus several critical steps are required. First, full length VirB2 propilin must be synthesized via the transcriptional activation of the *virB* operon and expression of *virB* gene products. Second, the recognition and cleavage of the signal peptidase cleavage site on the propilin generate a cleaved linear peptide product that is cyclized into the T pilin. Concomitantly, the transmembrane T-DNA transport apparatus comprised of VirB proteins is assembled. This transmembrane apparatus apparently also serves to transport T pilin since each *virB* gene is essential for T pilus biogenesis as an *A. tumefaciens* exocellular appendage [12].

How the T pilin subunits are assembled into the T pilus filament remains to be determined. If functional, the putative channel of about 2 nm diameter observed when T pili are treated with SDS (Fig. 1E) is of sufficient pore size to accommodate the T pilin for translocation to the growing T pilus tip. In flagella biogenesis, a channel of similar diameter is used to move flagellar structural components, including a muramidase to its growing tip; an export pathway that appears also to be used for the secretion of vir-

Table 1
Environmental effects on T pilus structure

Agent or condition[a]	Temperature (°C)	Morphology of pilus filament[b]	Presence of T pilin[c]
None added	37	flexuous	yes
Proteinase K (1 mg ml^{-1})	37	absence of pili	no
SDS (1%)	23	appearance of lumen	yes
Triton X-100 (1%)	23	vesicles present	yes
Urea (4 M)	23	flexuous	yes
pH 2.0 (10 mM HCl)	23	aggregate formation	yes
			pH 10 (1 mM NaOH)
23	flexuous	yes	
pH 13 (100 mM NaOH)	23	flexuous	yes
Glycerol (50%)	23	flexuous	yes
High temperature	70	beaded curls	yes

[a]T pili purified by velocity sedimentation in a linear sucrose gradient followed by density gradient centrifugation in CsCl. All treatments were 90 min incubation time.

[b]Each preparation was negatively stained with uranyl acetate and examined by transmission electron microscopy as described in Section 2.

[c]T pilin was detected by Western blots.

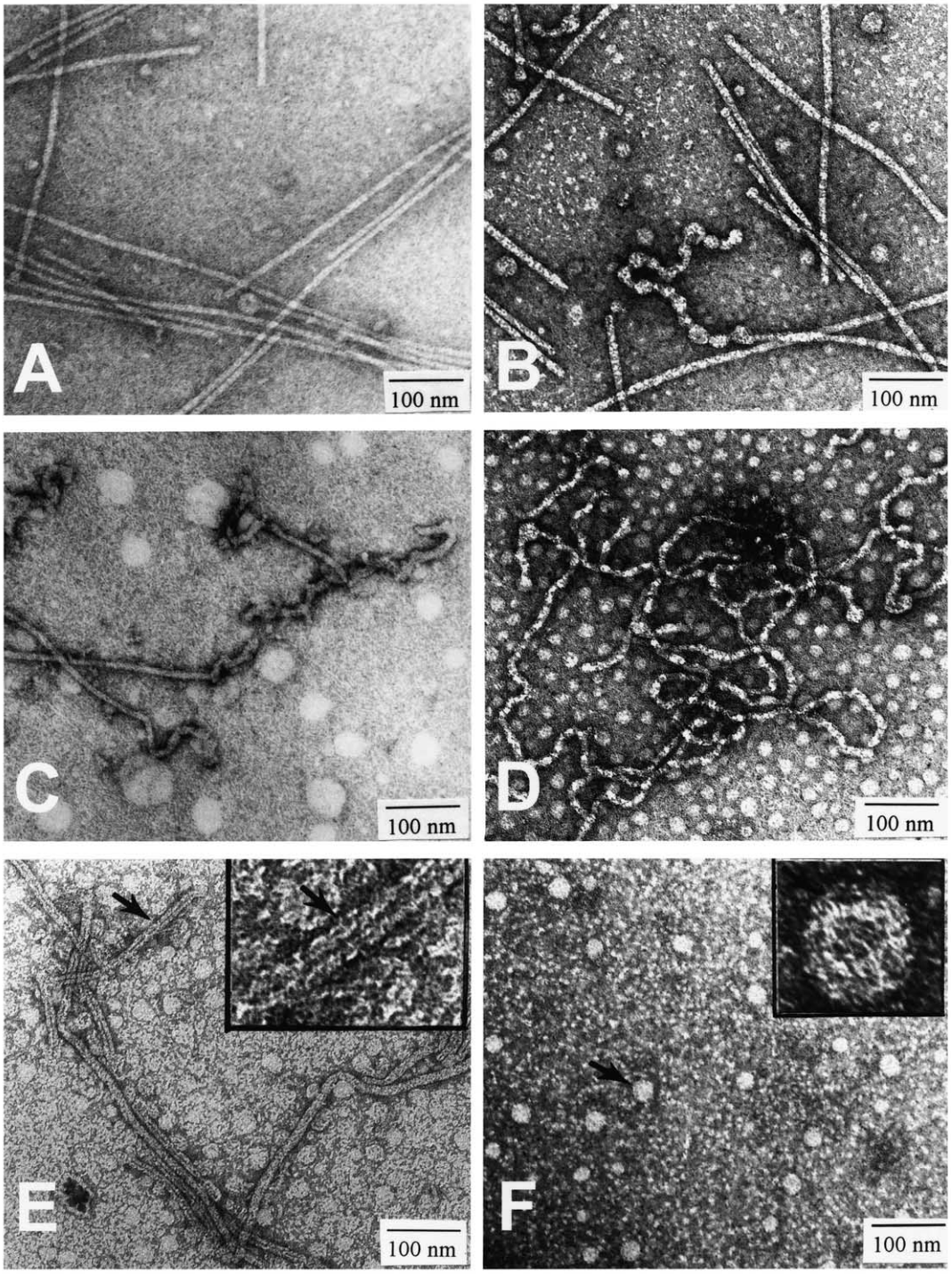

Fig. 1. Electron micrographs of CsCl-purified T pili subjected to various chemical and physical treatments. A: No treatment. B: pH 13 (0.1 N NaOH). C: 70°C, 10 min. D: pH 13 at 70°C. E: 0.1% sodium dodecyl sulfate. Inset: Magnified view of region shown by the arrow. F: 1% Triton X-100. Inset: Magnified view of sphere indicated by the arrow. Insets at 700 000× magnification.

ulence factors in type III export pathways [3,9,15]. Although we have not ruled out the possibility that T pili are generated from its base, translocation of T pilin subunits to the growing tip via the putative lumen could be the likely mechanism of T pilus assembly and extension. The Pap P pili are assembled from the base, which is anchored to the outer membrane [7], whereas the T pili are tightly associated with the cytoplasmic membrane like flagella [10,12]. Whichever model is the correct one might be elucidated by high-resolution electron microscopy of *A. tumefaciens* cells bearing T pili.

The results of our present studies indicate that T pili filaments are relatively resilient and therefore are likely to be durable organelles in the milieu of conditions occur-

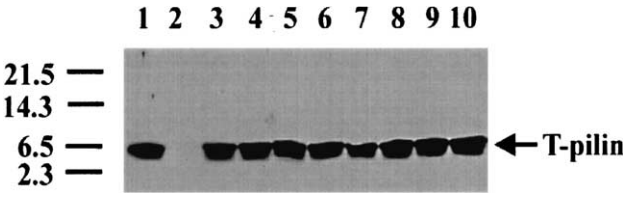

Fig. 2. Immunoblot analysis of T pili subjected to various treatments. Lanes: 1, no treatment; 2, proteinase K (1 mg ml^{-1}) at 37°C; 3, 0.1% sodium dodecyl sulfate at room temperature (RT); 4, 1% Triton X-100 at RT; 5, 4 M urea at RT; 6, pH 2 (0.01 N HCl); 7, pH 10 (1 mM NH_4OH); 8, pH 13 (0.1 N NaOH); 9, 50% glycerol; 10, 70°C, 10 min. Molecular masses of protein markers in kDa are indicated on the left. T pilin is marked by an arrow.

ring in microenvironments at the plant–bacterial interface. The T pilus appears to be a semi-rigid filament since high pH treatment at high temperature produces sinuous and relaxed filaments (Fig. 1D). Vesicle-like structures, which appear ring-shaped when viewed from their ends, are formed when T pili are treated with Triton X-100 (Fig. 1F). Vesicle-like structures have been observed on F pili termini when treated at pH 1.0 [2,4], and on filamentous phages treated with ethyl ether [16]. A 'knob-like' structure has been observed on one end of untreated T pili [17] and on the end of RP4 pili [5]. Isolation procedures and partial depolymerization of filamentous structures such as pili might result in the formation of vesicles. Folkhard et al. [6] have argued that vesicle formation suggests a mechanism of pilus retraction whereby pilin subunits might retract into a bolus of subunits; however, one can equally argue that the vesicle could represent the basal body of the pilus released by conditional excavation, i.e., mechanical dislodging from the membrane anchor. The reason for the presence of these vesicles remains unclear, but their existence favors the membrane anchor model.

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